CERTIFICATE OF MAILING
37 C.F.R. §1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date below:

February 13, 2002
Date

Steven L. Highlander

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shuyuan ZHANG *et al.*

Serial No.: 09/203,078

Filed: December 1, 1998

For: METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

Group Art Unit: 1648

Examiner: S. Foley

Atty. Dkt. No.: INRP:081

DECLARATION OF SHAWN GALLAGHER UNDER 37 C.F.R. §1.132

Hon. Commissioner for Patents
Washington, D.C. 20231

I, Shawn Gallagher, do declare that:

1. I am a citizen of the United States residing at 1730 Shoreline Drive, Missouri City, TX, 77459. I am an employee of Introgen Therapeutics, Inc. ("Introgen"), and a named inventor for the above-captioned application.

2. I have been employed by Introgen for 5 years and currently hold the position of Vice President of Product Development. My duties at Introgen include overseeing and management

of development of our adenoviral gene therapy products, one of which, adenoviral p53 ("Advexin®") is currently in phase III registration trials. I am also in charge of managing our clinical manufacturing facility, which is currently committed to the preparation of pharmaceutical grade adenoviral gene therapy products. I have extensive experience in adenovirus purification and pharmaceutical preparation, as evidenced by my attached *curriuculum vitae* [Exhibit 1].

3. In providing this declaration, I have reviewed the Office Action mailed on September 13, 2001. I am also familiar with the content of the above-captioned application, as well as with the pending claims.

4. Based on my reading of the Office Action, it appears that the examiner has misunderstood the present claims. The Action makes several references to the "cell cycle" in discussing the pending claims. However, there is no mention of the cell cycle in the pending claims but, rather, a recitation of "mid-log" and "stationary growth" phases. These terms refer to phases of ***a cell population growth curve***, and not to phases of the cell cycle, which are M (mitotic), G_1 (gap), S (synthesis) and G_2 (post-S, pre-M).[1] In this regard, the specification at pages 20-22, is instructive:

> In any cell culture system, there is a characteristic growth pattern following inoculation that includes a lag phase, an accelerated growth phase, an exponential or "log" phase, a negative growth acceleration phase and a plateau or stationary phase. The log and plateau phases give vital information about the cell line, the population doubling time during log growth, the growth rate, and the maximum cell density achieved in plateau. ***In the log phase, as growth continues, the cells reach their maximum rate of cell division.*** Numbers of cells increase in log relationship to time. During this period of

[1] Molecular Biology of the Cell, Alberts *et al.*, eds., Garland Publishing, NY, 1983, pp. 611-612 (Exhibit 2).

most active multiplication, the logarithms of the numbers of cells counted at short intervals, plotted against time, produce a straight line. By making one count at a specified time and a second count after an interval during the log phase of growth and knowing the number of elapsed time units, one can calculate the total number of cell divisions or doublings, and both the growth rate and generation time. Within a few hours or days after the commencement of the log phase, the rate of cell division begins to decline and some of the cells begin to die. This is reflected on the growth curve by a gradual flattening out of the line. ***Eventually the rate of cells dying is essentially equal to the rate of cells dividing, and the total viable population remains the same for a period of time. This is known as the stationary or plateau phase and is represented on the growth curve as a flattening out of the line where the slope approaches zero.***

... Typical growth curves are sigmoidal in shape, with the first part of the curve representing the lag phase, the center part of the curve representing the log phase, and the last part of the curve representing the plateau phase. The log phase is when the cells are growing at the highest rate, and as the cells reach their saturation density, their growth will slow and the culture will enter the plateau phase. A detailed description of cell culture techniques and theory can be found in Freshney, 1992 and Freshney, 1987.

An important aspect of the present invention is infection of the producer cells with recombinant adenovirus at an appropriate time to achieve maximal virus production. ***The inventors have found that maximal virus production is obtained when the producer cells are infected between about when the cells reach the first inflection point on the log phase of the cell growth curve, i.e., mid-log phase, and before the 2nd inflection point on the plateau phase of the cell growth curve, i.e. mid-plateau phase.*** This range can be determined easily for any cell type and any culture conditions with any cell culturing apparatus. The inflection points on a cell growth curve are when the shape of the line changes from a convex to a concave shape, or from a concave to a convex shape.

For most growth curves plotted on semi-log scales, the log phase of growth can be approximately represented by a linear increase in the slope of the line over time. That is, at any short interval between two points on the line of the logarithmic phase of the curve, the log of cell number is increasing in a linear fashion relative to time. ***Thus mid log phase can be approximately defined as the point or interval within the log phase in which the cells are dividing at their maximal rate, and the increase in logs of cell number is linear with respect to time. Late log phase can be defined as approximately the point or interval of time in which the rate of cell division has slowed, and the log of number of cells is no longer increasing in a linear fashion with respect to time. When looking at a growth curve, this area would be represented by gradual falling or flattening of the slope of the line. At early stationary phase, the rate of cell growth is decreasing and getting nearer the rate of cell death, and thus the slope of the line on the growth curve is even less than that at late log phase.*** At mid-stationary phase, the rate of cell growth is approximately equal to the rate of cell division and thus the line on the growth curve is relatively flat and has a slope approaching zero. It will be understood

that the skilled artisan can formulate growth curves for any such cell line and identify the aforementioned regions on the curve.

Specification at page 20, line 22, to page 22, line 27 (emphasis added). As illustrated by the highlighted portions, the terms "mid-log" and "stationary" phase have nothing to do with the cell cycle.

5. In light of the foregoing explanation of the terms "mid-log" and "stationary growth" phases, and based on factual information about the growth of cells in culture, it is my conclusion that the Huyghe *et al.* (1995; C44) paper, for the reasons discussed below, does not disclose our invention.

6. In the Huyghe *et al.* reference at page 1404, in the second paragraph under "MATERIALS AND METHODS," the article indicates that the cell monolayers were at "50-60% confluency" when infected with adenovirus. However, there is no information provided on the seeding density, lag phase or the doubling times for 293 cells under the authors' care, such that would permit a determination of whether these cells were in "mid-log" to "stationary growth" phases at the time the were infected – 2 to 2.5 days after seeding. Without knowledge of seeding density, lag phase or doubling times for the cells used in that study, there is no way one can conclude that Huyghe *et al.* infected the cells between mid-log and stationary phase.

7. At best, one can merely estimate the "phase" of the culture described in the Huyghe *et al.* reference, making certain assumptions and extrapolations, when infected at 2-2.5 days and 50-60% confluency. If one assumes that Huyghe *et al.* seeded at a seeding density of approximately

1-3 x 10^4 cells/cm^2, and that the 293 cells employed by Huyghe *et al.* had a lag phase of approximately 24 hours and a doubling time of approximately 36 hours, then one can calculate the phase at 50-60% confluency as early log phase, certainly less than mid-log phase, using the following calculations:

- **Initial density (midpoint of assumption range) = 2 x 10^4 cells/cm^2**
- **Growth period (2.5 days – lag time) = 1.5 days = 36 hours**
- **With a doubling time of 36 hours, the cells population will double once, giving final concentration equal to 2 x 10^4 cells/cm^2 x 2 = 4 x 10^4 cells/cm^2, consistent with early log phase density**

8. There are a number of scientific publications that support the foregoing conclusion. First, I would direct the examiner to Freshney, R.I., "Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed." [Exhibit 3]. At page 240, it is stated that "[t]oward the end of the log phase, the culture becomes confluent—*i.e.*, all the available growth surface is occupied and all the cells are in contact with surrounding cells." Based on this statement, it seems unlikely that Huyghe *et al.*'s cells, at 50-60% confluency, would be in late log phase.

9. In MediaTech's Technical Information bulletin [Exhibit 4], it is indicated, in the first paragraph of column 2 on the first page, that in order to ensure that cultures are in log phase, they must be at least 70% confluent. Thus, a culture that is only 50-60% confluent like that of Huyghe *et al.* is likely not in log phase, or at the very least, only in early log phase, not mid-log or late-log phase.

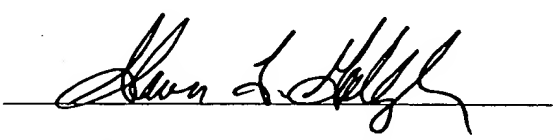
10. Next, I refer to the article of Kuchler, "Biochemical Methods in Cell Culture and Virology" [Exhibit 5]. At page 90, the lag phase, which precedes the log phase, is said to vary from 24 to 48 hours. Given that Huyghe *et al.* infected cells between 48-60 hours after seeding, Kuchler suggests that Huyghe *et al.*'s cells would be barely out of lag phase. While specific cell lines or strains of cell lines can demonstrate significant variation in characteristic doubling times and duration of lag phase, our own experience with 293 cells from various sources indicates that lag times of 24-48 hours are not uncommon after passaging.

11. Although none of the preceding evidence presents conclusive proof as to the precise point in the population growth curve at which Huyghe *et al.* infected cells, it is my opinion, based on the available evidence and my sample calculation, that the cells infected by Huyghe *et al.* were likely, at the very latest, in early log phase, and not within the "mid-log phase to stationary growth phase" as specified by the pending claims.

12. I hereby declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the referenced patent application or any patent issued thereon.

12 Feb 2002

Date



Shawn Gallagher, M.S.

SHAWN L. GALLAGHER
1730 Shoreline Drive
Missouri City, TX 77459
(281) 499-8588; e-mail: sggolfnut@aol.com

CAREER SUMMARY:

High energy, team player with an extensive history of successful program management in the following areas:

- cGMP manufacturing
- Facility design and construction
- Process development (fermentation and purification)
- Contract negotiation and management
- Strategic planning
- Validation

EXPERIENCE:

1996 to Present **INTROGEN THERAPEUTICS, INC.** **Houston, TX**
A leading biopharmaceutical company in the commercialization of gene based therapeutics.

Vice President, Product Development **1999-present**
Vice President, Manufacturing **1996-1999**

- Corporate Officer responsible for manufacturing and quality systems (QA/QC) for the cGMP production of viral vectors in support of cancer gene therapy clinical program.
- Directed the creation of a cGMP infrastructure to facilitate the transition of novel technology from an academic setting (at M.D. Anderson Cancer Center) to commercial development.
- Project Executive for the design of a new commercial manufacturing facility currently under construction.
- Lead the process engineering team in the development and implementation of a scalable manufacturing process for adenoviral based viral vectors.
- Primary technical liaison with pharmaceutical partner (RPR).

1995 to 1996 **MAGENTA CORPORATION** **Rockville, MD**
The foremost contract manufacturing organization servicing the gene therapy industry with the production of viral vectors for clinical use.

Director of Operations

- Coordinated the activities for two functional groups providing the gene therapy and broader biopharmaceutical industries with contract manufacturing services. These two groups, Virus Production and Cell Banking, occupy four dedicated cGMP manufacturing suites and apply

large scale mammalian and insect cell culture techniques to varied manufacturing problems.

- Interfaced with clients from initial contact phase through technology transfer until completion of production and testing.

- Provided technical support for bioreactor system development and worked with the management team to establish overall objectives and priorities for the process development group.
- Worked closely with Sales and Marketing staff to develop corporate advertising strategy and pricing programs.
- Member of parent company senior management taskforce charged with the development of a long term strategic plan and initiation of a corporate reengineering effort designed to align the company to meet the newly defined strategic goals.

1991 to 1995 **IMMUNOGEN, INC.** **Cambridge, MA**

A leader in the discovery and development of novel biopharmaceuticals for the treatment of cancer and immune system mediated diseases.

Assistant Director, Manufacturing **1993 to 1995**

- Directed all protein production activities in support of pre-clinical and clinical studies.
- Facilitated process optimization efforts as the manager of the cell culture development group.

Operations Manager, Manufacturing **1992 to 1993**

- Met commissioning timeline and exceeded budgetary goals as project manager during design, construction, start-up, and validation of a 31,000 square foot biopharmaceutical manufacturing facility.

Senior Bioprocess Engineer **1991 to 1992**

- Optimized and scaled-up the conjugation and purification of Antibody-Blocked Ricin immunotoxins and transferred process to manufacturing.

1987 to 1991 **BIOTECHNETICS, A BRUNSWICK COMPANY** **San Diego, CA**

A research and development organization involved in contract manufacturing of mammalian cell expressed proteins for academia and industry.

Bioproduction Manager **1989 to 1991**

Directed the mammalian cell processing team.

- Produced expressed proteins, under cGMP, at industrial scale.
- Provided technical direction and coordination for bioprocessing, tissue culture, and analytical chemistry sections.

Biochemical Engineer **1987 to 1989**

Responsible for the design and operational trouble-shooting of process equipment associated with mammalian cell bioreactor-based manufacturing.

- Developed or improved systems for high density cell culture (immobilized and suspension), continuous media make-up, sterilization, clarification, ultrafiltration, and chromatographic purification.
- Provided theoretical calculations, modeling, prototype fabrication, and pilot scale

support for production.

1985 to 1987 **UNIVERSITY OF CALIFORNIA AT SAN DIEGO** **La Jolla, CA**

Research Assistant /Teaching Assistant, Departments of Chemistry and AMES

EDUCATION:

MS, Chemical Engineering, University of California at San Diego, 1987.

- Investigation of the physiological behavior of mammalian cells.
- Design, testing, and modeling of novel hollow-fiber bioreactor systems.

BS, Chemical Engineering, University of Colorado, Boulder, 1985.

- Study of the growth kinetics of *Saccharomyces cerevisiae* in chemically defined media.

AWARD: 1989 Kirkpatrick Chemical Engineering Achievement Award.

BioTechnetics was an honor award recipient. This award is given every two years in recognition of outstanding chemical engineering technology, successfully commercialized and achieved through group effort. The biochemical engineering group was honored for the development of novel bioreactor systems. As a key member of the BioTechnetics process development team, I played an integral role in the successful commercialization of the bioreactor technology.

Chemical Engineering 96,12:79, 1989.

PROFESSIONAL AFFILIATIONS:

AIChE, ACS, ESACT, ISPE

REFERENCES:

Available on request

SHAWN L. GALLAGHER
1730 Shoreline Drive
Missouri City, TX 77459
(281) 499-8588; e-mail: sggolfnut@aol.com

PUBLICATIONS AND PRESENTATIONS

Wu, Z, Gallagher, S, Wilson, D, Zhang, S: **Development of a Lyophilization Process for Long Term Storage of an Adenoviral Vector.** Paper presented by Shuyuan Zhang at the Williamsburg Bioprocessing Conference, Williamsburg, VA: 16 November, 1998.

Gallagher, SL: **Process Validation: A Basis for Robust Clinical Development.** Presented at FDA/NCI Gene Therapy Conference, 1997, Bethesda, MD: 16 July 1997.

Gallagher, SL: **Getting Started in Gene Therapy Vector Development - Considerations for Process Development and Manufacturing.** Presented at FDA/NCI Gene Therapy Conference, 1996, Bethesda, MD: 12 July 1996.

Gallagher, SL: **Manufacturing Strategies for Rapid Access to the Clinic.** Presented at Business Development Strategies for Genomics and Gene Therapy Products, San Francisco, CA: 18 January 1996.

Gallagher, SL: **Contract Production of Viral Vectors for Gene Therapy.** Presented at Williamsburg Bioprocessing Conference, Williamsburg, VA: 6 November, 1995.

Gallagher, SL: **Virus Vectors for Gene Therapy Clinical Trials: Effective Communication and Project Management in Contract Manufacturing.** Presented at Contract Manufacturing '95, Arlington, VA: 14 September 1995.

Gallagher, SL: **Flexible Design Approaches for Multi-Product and Future Manufacturing Capacity.** Presented at BIO Eighth International Biotechnology Meeting, Toronto, Canada: 24 May 1994.

Gallagher, SL: **In-House Manufacturing or Contract Production: Critical Considerations.** Presented at BioEast '93, Washington, DC: 25 January 1993.

Hofmann, F, Gallagher, S: **Large Scale Production of Pharmaceutical Grade Monoclonal Antibodies.** In Groves MJ, Olson W (eds): **"Sterile Pharmaceutical Manufacturing: Applications for the 1990's - Volume II."** Buffalo Grove, IL: Interpharm Press, 93, 1991.

Gallagher, SL (invited): **Continuous Production of Expressed Proteins Utilizing a Flat Sheet Membrane Bioreactor.** BioEast '91, Washington, DC: 7 January 1991.

Handgretinger, R, Dopfer, R, Bader, P, Klingebeil, TH, Mueller, BM, Reisfeld, RA, Gallagher, S, Rueland, P, Koscielnak, E, Treuner, J, Niethammer, D: **Treatment of Neuroblastoma with the Murine anti-GD2 Antibody 14G2a.** Poster presented at Society for Biological Therapy, 1990 Annual Meeting (Immunotherapy of Cancer), San Diego, CA: 10-13 November 1990.

Hofmann, F, Wrasidlo, W, deWinter, D, Gallagher, S: **Fully Integrated, Compact Membrane Reactor System for the Large Scale Production of Monoclonal Antibodies.** In Spier, RE, Griffiths, JB, Stephenne, J, Crooy, PJ (eds): **"Advances in Animal Cell Biology and Technology for Bioprocesses."** London: Butterworth & Co., 305, 1989.

Gallagher, SL: **Novel, Compact, Membrane-based Reactor System for the Large-scale Production of Mammalian Cell Products**. Presented at the Southern California Biotechnology Symposium, San Diego, CA: 17 June 1988.

Tharakan, JT, Gallagher, SL, Chau, PC: **Hollow Fiber Bioreactors for Mammalian Cell Culture**. Advances in Biotechnological Processes 7:153, 1988.

Gallagher, SL, Tharakan, JT, Chau, PC: **An Intercalated-spiral Wound Hollow Fiber Bioreactor for the Culture of Mammalian Cells**. Biotechnology Techniques 1:91, 1987.

PATENT APPLICATIONS

Zhang, S, Thwin, C, Wu, Z, Cho, T, Gallagher, S: **An Improved Method for the Production and Purification of Adenoviral Vectors.**
Filed: 20 November 1996.

Hofmann, F, deWinter, D, Gallagher, S: **Continuous Bioreactor Product Concentration System.**
Filed: 31 May 1988.

Hofmann, F, deWinter, D, Gallagher, S: **Gas Pressure Displacement Bioreactor.**
Filed: 19 April 1988.

RELATED EXPERIENCE AND EDUCATION

1994-96 Admitted to the MBA programs at Babson Graduate School of Business and University of Maryland.

1993-94 **Instructor:** "Fundamentals of Engineering for Biotechnology" (MED BT 270S) during summer sessions at Program in Biomedical Laboratory and Clinical Sciences, Boston University School of Medicine, Metropolitan College.

MOLECULAR BIOLOGY OF THE CELL

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson

Garland Publishing, Inc.
New York & London

"Long ago it became evident that the key to every biological problem must finally be sought in the cell, for every living organism is, or at sometime has been, a cell."

Edmund B. Wilson
The Cell in Development and Heredity
3rd edition, 1925, Macmillan, Inc.

Bruce Alberts received his Ph.D. from Harvard University and is currently a Professor in the Department of Biophysics and Biochemistry at the University of California Medical School in San Francisco. *Dennis Bray* received his Ph.D. from the Massachusetts Institute of Technology and is currently a Senior Scientist in the Medical Research Council Cell Biophysics Unit at King's College London. *Julian Lewis* received his D.Phil. from Oxford University and is currently a Lecturer in the Anatomy Department at King's College London. *Martin Raff* received his M.D. degree from McGill University and is currently a Professor in the Zoology Department at University College London. *Keith Roberts* received his Ph.D. from Cambridge University and is currently Head of the Department of Cell Biology at the John Innes Institute, Norwich. *James D. Watson* received his Ph.D. from the University of Indiana and is currently Director of the Cold Spring Harbor Laboratory. He is the author of *Molecular Biology of the Gene* and, with Francis Crick and Maurice Wilkins, won the Nobel Prize in Medicine and Physiology in 1962.

Cover photograph kindly provided by Michael Verderame and Robert Pollack of Columbia University. The fluorescein-phalloidin used to stain the actin cables was the generous gift of Drs. Theodor Wieland and A. Deboben of the Max Planck Institute, West Germany. The photograph is of a mouse fibroblast that had been transformed to anchorage-independent growth by the virus Simian Virus 40 (SV40) and subsequently selected for anchorage-dependent growth. This particular cell was stained for SV40 large T antigen (*red*) and fluorescein-phalloidin (*green*), which specifically stains F actin.

Library of Congress Cataloging in Publication Data
Main entry under title:

Molecular biology of the cell.

Includes bibliographies and index.
1. Cytology. 2. Molecular biology. I. Alberts, Bruce, 1938– . [DNLM: 1. Cells. 2. Molecular biology. QH 581.2 M718]
QH581.2.M64 1983 574.87 82-15692
ISBN 0-8240-7282-0

Published by Garland Publishing, Inc.
136 Madison Avenue, New York, NY 10016

Printed in the United States of America

15 14 13 12 11 10 9 8 7 6 5 4 3 2

Cell Growth and Division

11

As highly organized units in a universe favoring disorder, cells are subject to wear and tear as well as to accidents. Any individual cell is therefore bound to die. If an organism is to continue to live, it must create new cells at a rate as fast as that at which its cells die. For this reason, cell division is central to the life of all organisms. In an adult human, for example, millions of cells must divide every second simply to maintain the status quo.

The process of cell division itself is strikingly visible in the microscope; it consists of two sequential processes: nuclear division (called **mitosis**) and cytoplasmic division (called **cytokinesis**). But before a typical cell can divide, it must double its mass and duplicate all of its contents. Only in this way will the two new daughter cells contain all of the components that they need to begin their own cycle of cell growth followed by division. Most of the work involved in preparing for division goes on invisibly during the growth phase of the cell cycle, which is, quite misleadingly, denoted as **interphase**.

Although a cell spends most of its lifetime in interphase and only occasional periods in the cell-division phase, most early work on the cell cycle focused on the brief division events (mitosis and cytokinesis), largely because they could be studied by direct microscopic examination. More recently, through the use of more indirect and sophisticated techniques, we have learned a considerable amount about the interphase part of the cell cycle as well. In this chapter we shall describe some of the methods currently used to study the cell cycle, consider cell-cycle regulation, and discuss several of the main events occurring during each of its different phases. Although our knowledge of the molecular basis of the cell cycle is fragmentary, wherever possible we shall try to discuss the mechanisms that are likely to be involved.

The Control of Cell Division[1,2]

Most cell components are made continuously throughout the interphase period between cell divisions. It is, therefore, difficult to define distinct stages in the progression of the growing cell through interphase. One outstanding exception is DNA synthesis, since the DNA in the cell nucleus is replicated only

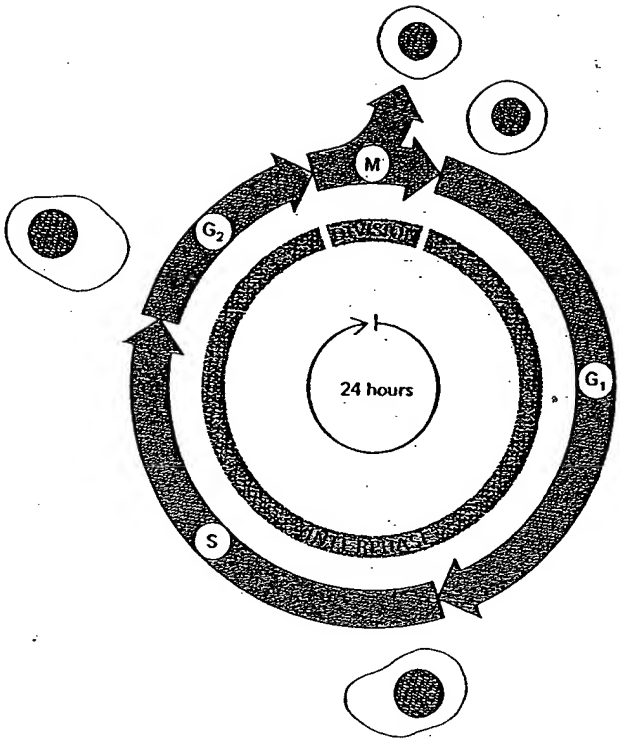


Figure 11–1 The four successive phases of the cell cycle. After the *M phase*, which consists of nuclear division (mitosis) and cytoplasmic division (cytokinesis), the daughter cells begin interphase of a new cycle. Interphase starts with the *G_1 phase*, in which the cells, whose biosynthetic activities have been greatly slowed during mitosis, resume a high rate of biosynthesis. The *S phase* begins when DNA synthesis starts, and ends when the DNA content of the nucleus has doubled and the chromosomes have replicated (each chromosome is now said to consist of two identical "sister chromatids"). The cell then enters the *G_2 phase*, which ends when mitosis starts. The M phase begins with mitosis (hence the "M") and ends with cytokinesis. During the early part of the M phase, the replicated chromosomes condense from their extended interphase condition and are easily seen in the light microscope. The nuclear envelope breaks down, and each chromosome undergoes precisely orchestrated movements that result in the separation of its pair of sister chromatids as the nuclear contents are divided. Two new nuclear envelopes then form, and the cytoplasm divides to generate two daughter cells, each with a single nucleus. This process of cytokinesis terminates the M phase and marks the beginning of the interphase of the next cell cycle. Although a typical 24-hour cycle is illustrated here, cell-cycle times in higher eucaryotic cells vary widely, with most of the variability being in the length of the G_1 phase (see text).

during a limited portion of interphase. This period is denoted as the **S phase** (S = synthesis) of the cell cycle. The other distinct stage of the cycle is, of course, the cell-division phase, which includes both nuclear division (mitosis) and the cytoplasmic division (cytokinesis) that follows. The entire cell-division phase is denoted as the **M phase** (M = mitotic). This leaves the period between the M phase and the start of DNA synthesis, which is called the **G_1 phase** (G = gap), and the period between the completion of DNA synthesis and the next M phase, which is called the **G_2 phase.** Interphase is thus composed of successive G_1, S, and G_2 phases, and it normally comprises 90% or more of the total cell cycle time. For example, in rapidly dividing cells of higher eucaryotes, the successive cell divisions (M phases) that interrupt interphase generally occur only once every 16 to 24 hours, and each M phase itself lasts only 1 to 2 hours. A typical cell cycle with its four successive phases is illustrated in Figure 11–1, and some of the major sequential events are outlined in the legend.

The Cells in a Multicellular Organism Divide at Very Different Rates[3]

In unicellular organisms, such as bacteria and protozoa, there is a strong selective pressure for each individual cell to grow and divide as rapidly as possible. For this reason the rate of cell division is generally limited only by the rate at which nutrients can be taken up from the medium and converted to cellular materials. The situation in multicellular animals is quite different. To varying degrees, different cell types have given up their potential for rapid division so that their numbers can be kept at a level that is optimal for the organism as a whole: it is the survival of the organism that is paramount, not the survival of any of its individual cells. As a result, the 10^{13} cells of the human body divide at very different rates. Some cells, such as neurons, skeletal-muscle cells, and red blood cells, do not divide at all once they are mature. Other

Second Edition

CULTURE OF ANIMAL CELLS

A Manual of Basic Technique

R. Ian Freshney

Department of Medical Oncology
Cancer Research Campaign Laboratories
University of Glasgow

Alan R. Liss, Inc., New York

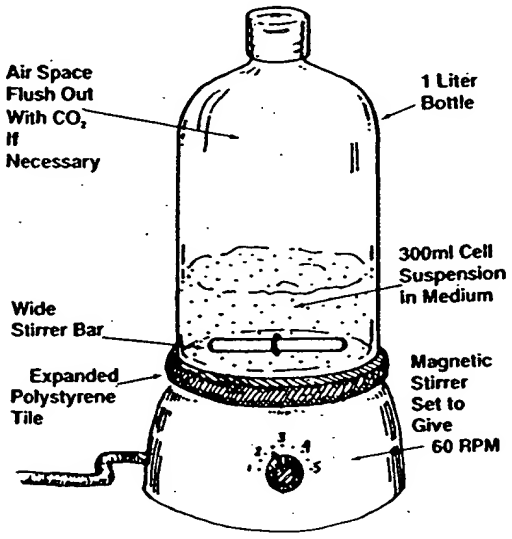


Fig. 10.2. *Simple stirrer culture for cells growing in suspension. An expanded polystyrene mat (dark shaded area below bottle) should be interposed between the bottle and the magnetic stirrer to avoid heat transfer from the stirrer motor.*

Cell morphology. When checking a culture for routine maintenance, be alert to signs of morphological deterioration: granularity around the nucleus, cytoplasmic vacuolation, and rounding up of the cells with detachment from the substrate (Fig. 10.1). This may imply that the culture requires a medium change, or may indicate a more serious problem, e.g., inadequate or toxic medium or serum, microbiological contamination, or senescence of the cell line. During routine maintenance, the medium change or subculture frequency should prevent such deterioration.

Volume, Depth, and Surface Area

The usual ratio of medium volume to surface area is 0.2–0.5 ml/cm^2. The upper limit is set by gaseous diffusion through the liquid layer and the optimum will depend on the oxygen requirement of the cells. Cells with a high O_2 requirement will be better in shallow medium (2 mm) and those with a low requirement may do better in deep medium (5 mm). If the depth is

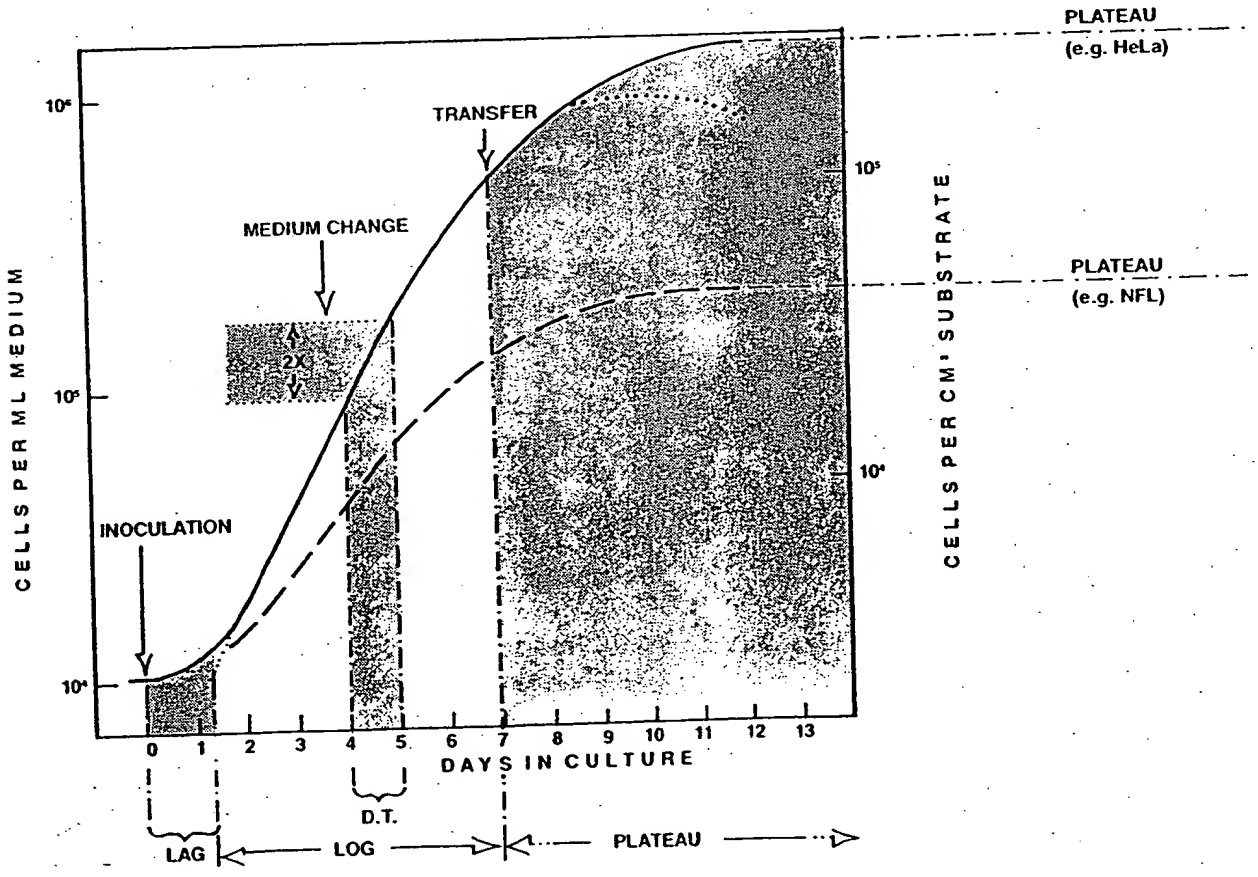


Fig. 10.3. *Diagram of growth curve of a continuous cell line such as HeLa and a finite cell line, NFL (normal fetal lung fibroblasts). The solid line represents growth of HeLa; the dashed; line illustrates the lower plateau obtained with NFL (see text).*

GROWTH CYCLE

As described in Chapter 10, following subculture, cells will progress through a characteristic growth pattern of lag phase, exponential or "log" phase, and stationary or "plateau" phase (see Fig. 10.3). The log and plateau phases give vital information about the cell line, the population doubling time during log growth, and the maximum cell density achieved in plateau (saturation density). Measurement of the population doubling time is used to quantify the response of the cells to different inhibitory or stimulatory culture conditions such as variations in nutrient concentration, hormonal effects, or toxic drugs. It is also a good monitor of the culture during serial passage and enables the calculation of cell yields and the dilution factor required at subculture.

It must be emphasized that the population doubling time is an average figure and describes the net result of a wide range of division rates, including zero, within the culture.

Single time points are unsatisfactory for monitoring growth, without knowing the shape of the growth curve. A reduced cell count after, say, 5 days could be caused by a reduced growth rate of some or all the cells, a longer lag period implying adaptation or cell loss (difficult to distinguish), or a reduction in saturation density. This is not to say that growth curves are of no value. They can be useful for a rapid screen, and once the response being monitored is fully characterized and the type of response predictable, e.g., an increased doubling time, then single time point observations may be sufficient. Growth curves are particularly useful for the determination of saturation density, although growth at saturation density should be assessed by the labeling index with ^{3}H-thymidine (see below and Chapter 15).

The preferred method for analyzing growth and survival at lower cell densities is by clonal growth analysis (see Chapter 19). This technique will reveal differences in growth rate within a population and will distinguish between alterations in growth rate (colony size) and survival (colony number). It should be remembered, however, that cells may grow differently as isolated colonies at low cell densities. Fewer cells will survive even under ideal conditions, and all interaction is lost until the colony starts to form. Heterogeneity in clonal growth rates reflects differences in growth capacity between lineages within a population, but these need not necessarily be expressed in an interacting monolayer at higher densities where cell communication is possible.

The population doubling time (PDT) derived from a growth curve should not be confused with the cell cycle or generation time. The PDT is an average figure for the population and subject to the reservations stated above. The cell cycle time or generation time is measured from one point in the cell cycle until the same point is reached again (see below) and refers only to the growing cells in the population, while the PDT is influenced by nongrowing and dying cells. PDTs vary from 12–15 hr in rapidly growing mouse leukemias like the L1210, to 24–36 hr in many adherent continuous cell lines, and up to 60 or 72 hr in slow-growing finite cell lines.

A growth cycle is performed each time the culture is passaged and can be analyzed in more detail as described below.

Outline

Set up a series of cultures at three different cell concentrations and count the cells at daily intervals until they "plateau."

Materials

Cell culture
24-well plates (sterile)
100 ml growth medium (sterile)
0.25% crude trypsin (for monolayer cultures only) (sterile)
plastic box to hold plates
CO_2 incubator or CO_2 supply to gas box

Protocol

1.
Trypsinize cells as for regular subculture (see above).
2.
Dilute cell suspension to 10^5 cells/ml, 3×10^4 cells/ml, and 10^4 cells/ml, in 25 ml medium for each concentration.
3.
Seed three 24-well plates, one at each cell concentration, with 1 ml per well. Add cell suspension slowly from the center of the well so that it does not swirl around the well. Similarly, do not shake the plate to mix the cells, as the circular movement of medium will concentrate the cells in the middle of the well.
4.
Place in a humid CO_2 incubator or sealed box gassed with CO_2.
5.
After 24 hr, remove plates from incubator and

count the cells in three wells of each plate: (a) remove medium completely from wells to be counted; (b) add 1 ml trypsin to each well; (3) incubate with trypsin; and (4) after 15 min, disperse cells in trypsin and transfer 0.4 ml to counting fluid and count on cell counter.

Note. Hemocytometer counting may be used but may be difficult at lower cell concentrations. Reduce trypsin volume to 0.1 ml and disperse cells carefully without frothing using a micropipette and transfer to hemocytometer.

6.

Return plate to incubator as soon as cell samples in trypsin are removed. The plate must be out of the incubator for the minimum length of time, to avoid disruption of normal growth.

7.

Repeat sampling at 48 and 72 hr as in steps 5 and 6.

8.

Change medium at 72 hr or sooner if indicated by pH drop (see above).

9.

Continue sampling at daily intervals for rapidly growing cells (doubling time 12–14 hr) but reduce frequency of sampling to every 2 days for slowly growing cells (doubling time > 24 hr), until plateau is reached.

10.

Keep changing medium every 1, 2, or 3 days as indicated by pH.

Analysis

1. Calculate cell number per well, per ml of culture medium (same figure), and per cm^2 of available growth surface in well. (Stain one or two wells (see Chapter 13) at each density to determine whether distribution of cells in wells is uniform and whether they grow up the sides of the well.)

2. Plot cell density (per cm^2) and cell concentration (per ml), on a log scale, against time on a linear scale (Fig. 10.3).

3. Determine the lag time, population doubling time, and plateau density (see below and Fig 10.3)

4. Establish which is the appropriate starting density for routine passage. Repeat growth curve at intermediate cell concentrations if necessary

Variations

1. Different culture vessels may be used, e.g., 25 cm^2 flasks, although more cells and medium will be required, or flat-bottomed glass-sample tubes. Individual tubes have the advantage that the rest are not disturbed when samples are removed for counting.

2. Frequency of medium changing may be altered.

3. Different media or supplements may be tested.

Suspension cultures

1.

Add cell suspension in growth medium to wells at a range of concentrations as for monolayer.

2.

Sample 0.4 ml at intervals as per trypsin samples. Alternatively, seed two 75-cm^2 flasks with 20 ml for each cell concentration and sample 0.4 ml from each flask daily or as required. Mix well before sampling and keep flasks out of incubator for the minimum length of time. Do not feed cultures during growth curve.

The growth cycle (Fig. 10.3) is conventionally divided into three phases.

The Lag Phase

This is the time following subculture and reseeding during which there is little evidence of an increase in cell number. It is a period of adaptation during which the cell replaces elements of the glycocalyx lost during trypsinization, attaches to the substrate, and spreads out. During spreading the cytoskeleton reappears and its reappearance is probably an integral part of the spreading process. Enzymes, such as DNA polymerase, increase, followed by the synthesis of new DNA and structural proteins. Some specialized cell products may disappear and not reappear until cessation of cell proliferation at high cell density.

The Log Phase

This is the period of exponential increase in cell number following the lag period and terminating one or two doublings after confluence is reached. The length of the log phase depends on the seeding density, the growth rate of the cells, and the density at which cell proliferation is inhibited by density. In the log phase the growth fraction is high (usually 90–100%) and the culture is in its most reproducible form. It is the optimal time for sampling since the population is at its most uniform and viability is high. The cells are, however, randomly distributed in the cell cycle and, for some purposes, may need to be synchronized (see Chapter 23).

The Plateau Phase

Toward the end of the log phase, the culture becomes confluent—i.e., all the available growth surface is occupied and all the cells are in contact with surrounding cells. Following confluence the growth rate of the culture is reduced, and in some cases, cell proliferation ceases almost completely after one or two further population doublings. At this stage, the culture enters the plateau (or stationary) phase, and the growth fraction falls to between 0 and 10%. The cells may become less motile; some fibroblasts become oriented with respect to one another, forming a typical parallel array of cells. "Ruffling" of the plasma membrane is reduced, and the cell both occupies less surface area of substrate and presents less of its own surface to the medium. There may be a relative increase in the synthesis of specialized versus structural proteins and the constitution and charge of the cell surface may be changed.

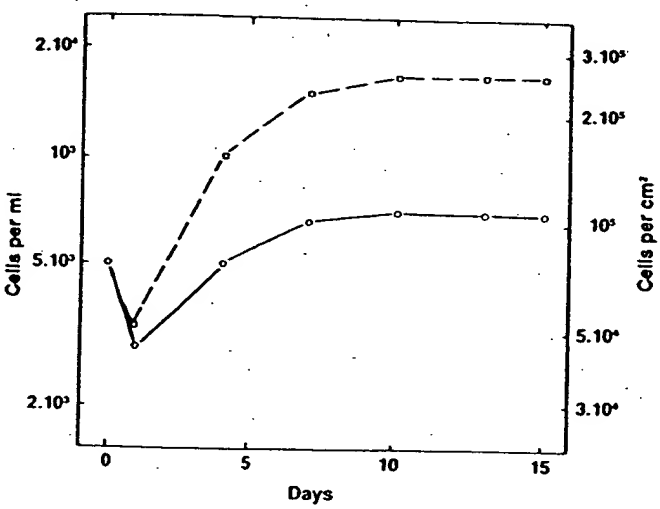


Fig. 18.5. *Difference in plateaus (saturation densities) attained by cultures from normal brain (circles, solid line) and a glioma (squares, broken line). Cells were seeded onto 13-mm coverslips and 48 hr later the coverslips were transferred to 9-cm petri dishes with 20 ml growth medium, to minimize exhaustion of the medium.*

The phenomenon of cessation of motility, membrane ruffling, and growth was described originally by Abercombie and Heaysman [1964] and designated "*contact inhibition.*" It has since been realized that the reduction of the growth of normal cells after confluence is reached is not due solely to contact but may also involve reduced cell spreading [Stoker et al., 1968; Folkman and Moscona, 1978], depletion of nutrients, and, particularly, growth factors [Stoker, 1973; Dulbecco and Elkington, 1973; Westermark and Wasteson, 1975] in the medium [Holley et al., 1978]. The term "*density limitation*" of growth has, therefore, been used to remove the implication that cell-cell contact is the major limiting factor [Stoker and Rubin, 1967], and "contact inhibition" is best reserved for those events directly contingent on cell contact, i.e., reduced cell motility and membrane activity, resulting in the formation of a strict monolayer and orientation of the cells with respect to each other.

Cultures of normal simple epithelial and endothelial cells will stop growing after reaching confluence and remain as a monolayer. Most cultures, however, with regular replenishment of medium, will continue to proliferate, although at a reduced rate, well beyond confluence, resulting in multilayers of cells. Human embryonic lung, or adult skin, fibroblasts, which express contact inhibition of movement, will continue to proliferate, laying down layers of collagen between the cell layers, until multilayers of six or more cells can be reached under optimal conditions [Kruse et al., 1970]. They still retain an ordered parallel array, however. The terms "plateau" and "stationary" are not strictly accurate, therefore, and should be used with caution.

Cultures which have transformed spontaneously or have been transformed by virus or chemical carcinogens will usually reach a higher cell density in the plateau phase than their normal counterparts [Westermark, 1974] (Fig. 18.5). This is accompanied by a higher growth fraction and loss of density limitation. These cultures are often *anchorage independent* for growth—i.e., they can easily be made to grow in suspension (see Density Limitation of Growth in Chapter 15; also see Chapter 2).

The construction of a growth curve from cell counts performed at intervals after subculture enables the measurement of a number of parameters which should be found to be characteristic of the cell line under a given set of culture conditions. The first of these is the duration of the *lag period* or "lag time" obtained by extrapolating a line drawn through the points on the exponential phase until it intersects the seeding or inoculum concentration (see Fig 10.3), and reading off the elapsed time since seeding equivalent to that intercept. The second is the *doubling time*, i.e., the time taken for the culture to increase two-fold in the middle of the exponential, or "log," phase of growth. This should not be confused with the *generation time* or *cell cycle time* (see below), which are determined by measuring the transit of a population of cells through the

cell cycle until they return to the same point in the cell cycle.

The last of the commonly derived measurements from the growth cycle is the "*plateau level*" or "*saturation density*." This is the cell concentration in the plateau phase and is dependent on cell type and frequency of medium replenishment. It is difficult to measure accurately as a steady state is not achieved as easily as in the log phase. Ideally the culture should be perfused; but a reasonable compromise may be achieved by growing the cells on a restricted area, say a small-diameter coverslip (15 mm) in a large-diameter petri dish (90 mm) with 20 ml of medium replaced daily (see Chapter 15). Under these conditions, medium limitation of growth is minimal, and cell density exerts the major effect. Counting the cells under these conditions gives a more accurate and reproducible measurement. "Plateau" does not imply complete cessation of cell proliferation but represents a steady state where cell division is balanced by cell loss.

With normal cells a "steady state" may be achievable by not replenishing the growth factors in the medium. In this case cells are seeded and grown and plateau reached without changing the medium. Clearly, the conditions used to attain "plateau" must be carefully defined.

The maximum cell concentration in suspension cultures, which are not limited by available substrate, is usually limited by available nutrients. By fortifying the medium with a higher concentration of amino acids, Pirt and others [Birch and Pirt, 1971; Blaker et al., 1971] were able to obtain a maximum cell concentration of 5×10^6 cells/ml for L"S" cells, far in excess of what can be achieved with attached cells.

PLATING EFFICIENCY

When cells are plated out as a single cell suspension at low cell densities (2–50 cells/cm^2), they will grow as discrete colonies (see Chapter 11). When these are counted the results are expressed as the plating efficiency:

$$\frac{\text{No. of colonies formed}}{\text{No. of cells seeded}} \times 100 = \text{plating efficiency.}$$

If it can be confirmed that each colony grew from a single cell, this term becomes the *cloning efficiency*.

Strictly according to the definition, plating efficiency measurements are derived from counting colonies over a certain size (usually 16–50 cells) growing from a low inoculum of cells, and the term should not be used for the recovery of adherent cells after seeding at higher cell densities (e.g., 2×10^4 cells/cm^2). This is more properly called the *seeding efficiency*:

$$\frac{\text{No. of cells recovered}}{\text{No. of cells seeded}} \times 100 = \text{seeding efficiency.}$$

It should be measured at a time when the maximum number of cells has attached but before mitosis starts. This provides a crude measurement of recovery in, for example, routine cell freezing or primary culture.

CLONAL GROWTH ASSAY BY DILUTION CLONING

The protocol for dilution cloning [Puck and Marcus, 1955] is given in Chapter 11. When colonies have formed, remove medium, rinse carefully in BSS, and fix and stain colonies (see Chapter 13).

Analysis

1. Count colonies and calculate plating efficiency. Magnifying viewers (e.g., Fig. 18.6) help to make counting easier.
2. The size distribution of the colonies may also be determined (e.g., to assay the growth promoting ability of a test medium or serum, see Chapter 7) by counting the number of cells per colony or by densitometry. Fix in 1% glutaraldehyde, stain the colonies with crystal violet, and measure absorption on a densitometer [McKeehan et al., 1977].

Automatic Colony Counting

If the colonies are uniform in shape and quite discrete, they may be counted on an automatic *colony counter* (e.g., New Brunswick, Artek, Micromeasurements Ltd.) which scans the plate with a conventional TV camera and analyzes the image to give an instantaneous readout of colony number. A size discriminator gives size analysis based on colony diameter (not always proportional to cell number, as cells may pile up in the center of the colony).

Though expensive, these instruments can save a great deal of time and make colony counting more objective. They will not cope well with colonies which overlap, or have irregular outlines.

LABELING INDEX

If a culture is labeled with [^{3}H]-thymidine, cells that are synthesizing DNA will incorporate the isotope.

Guide to Subculturing Cell Cultures

Morphology Each cell line or cell type expresses different characteristics in terms of growth and appearance in culture. Many cell lines grow as a single sheet monolayer attached to both themselves, and the culture vessel. Other cell types exist as single cells or clumps of cells suspended in the growth medium. Both adherent and suspension cultures must be maintained regularly to prevent overgrowth and accelerated cell death from exhausted medium and to promote the growth of the next generation of cells.

Cell Dissociation Viable subcultures may be obtained by transferring a particular volume of cells to new culture vessels with fresh medium. These fresh cultures are allowed to grow and divide as normal until such time the culture reaches confluence and the cells are used for experiments or subcultured. To do this correctly, obtain a single-cell suspension first. For adherent cell types, proteolytic enzymes, such as trypsin (MT Catalog Numbers 25-050, 25-052, 25-053, and 25-054), are used to break cell-cell and cell-substrate bonds and create a suspension from which new cultures may be split. For cultures already growing in suspension, this enzyme step is not necessary.

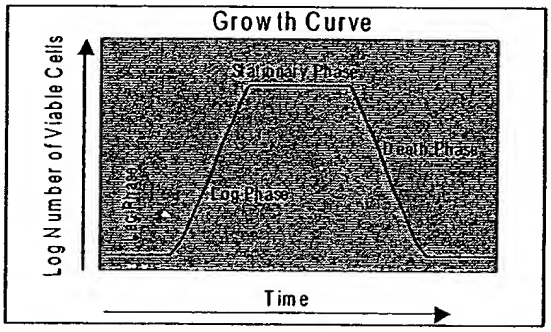
Harvesting describes the detachment of adherent cell lines to prepare a cell suspension for counting. During this step, the intercellular and intracellular (cell-substrate) bonds are broken, allowing the cells to separate into a single cell suspension. Depending on the cell type and the culture environment, this is achieved by using enzymatic or non-enzymatic dissociation solutions, such as Cellstripperâ , catalog number 25-056.

Growth Phases Cell growth typically exhibits a consistent pattern comprising three main phases, including an initial lag phase, a period of logarithmic growth, and a final stationary phase. See Figure 1. This growth pattern continues for each subculture despite the cell type. The initial lag phase occurs at the beginning of a subculture as the cells become accustomed to the new environment, during which they do not divide. The length of the log phase is determined by cell conditions prior to subculturing, as well as the seeding density and changes in the growth medium. The log (logarithmic) phase is a period of active proliferation, during which the number of cells increases exponentially. The length of the log phase is determined by many factors, including the seeding density and rate of normal cell growth, as well factors affecting the lag phase. The final stationary phase occurs when the rate of cell proliferation slows down. During this phase, the rate of cell division may be balanced by the rate of cell death, thus showing no change in cell density.

Subculturing is usually performed during the log phase when the cells are at their healthiest and are able to adapt to the new environment most efficiently. This is also the best time for cryopreservation and functionality studies. Check for cultures that appear at least 70% confluent.

Culture Examination Before handling, it is good practice to observe cultures both microscopically and macroscopically. Visual observations of the culture flask and medium may indicate evidence of microbial contamination including pH fluctuations and turbidity, as well as fungal colonies. The monolayer may also be viewed macroscopically to obtain a general idea of confluence. See Figures 2 and 3 below. This is most easily performed by viewing the culture vessel against a light source. Further microscopic observation may substantiate abnormal cell appearance and confirm microbial contamination. Rounding cells may indicate mitosis, especially if the cells are very refractive, or bright. Dead cells do not express this same brightness.

Figure 1. Growth Phases of Cells in Culture



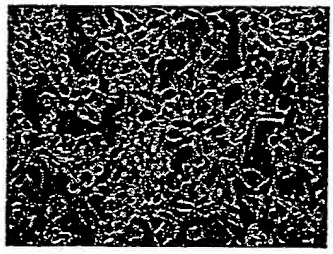


Figure 2. Monolayer
Confluent Monolayer. This culture is ready for splitting.

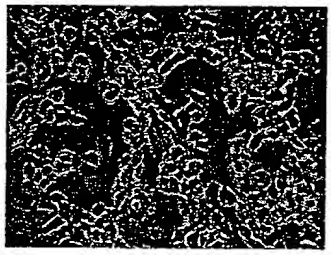


Figure 3. Monolayer
This monolayer appears less confluent than the monolayer in Figure 1. A cell count would determine if maximum cell density has been reached.

Routine Subcultivation & Maintenance

Procedure The following procedure describes the basic principles involved in the routine subcultivation and maintenance of cell cultures. **It is imperative that aseptic technique is maintained throughout this procedure.**

1. Visually examine cultures, as discussed above.

2. Harvest adherent cell lines using a dissociating agent. For suspension lines, this step is not required. Proceed to Step 7.

3. Aspirate and discard the culture medium.

4. Rinse the monolayer with the dissociation solution or a buffered salt solution without calcium and magnesium. This rinsing step removes any residual serum from the monolayer that could inactivate the trypsin. Use about 3ml for 25-cm^2 flasks and 5ml for 75-cm^2 flasks. Be sure to add the solution on the side of the flask opposite the monolayer to prevent cell loss. Swirl the solution gently across the cell sheet. Remove and discard the solution.

5. Repeat the washing step, this time using the dissociation solution. For more fastidious cell lines, the flask can be placed in a 37°C incubator to facilitate the enzyme. The flask may also be observed microscopically to monitor progress and prevent over-exposure of the cells to the enzyme activity. Non-enzymatic solutions, such as Cellstripperâ , will require longer incubation times than when using enzymes like trypsin, but are more gentle to the cells.

6. Once the cells are detached, add the desired amount of growth medium to the flask, creating a cell suspension. When pipetting the growth medium into the flask, be sure to "wash" the sides of the flask to ensure all cells become suspended. It is not unusual for a small amount of cells to remain attached to the flask or substrate. However, more vigorous pipetting may be necessary to break up cell clumps or to aid in the removal of attached cells.

7. Using the cell suspension, determine the appropriate inoculum for subculturing the particular cell line. This seeding density may be determined by performing growth rate studies, or by counting. When simply passing a culture, when tracking exact cell densities in not necessary, a split according to a suspension ratio is commonly used. For example, a 1:2 ratio indicates that the cell suspension may be split in half between two culture flasks of equal surface area. Counting, however, requires a hemacytometer or other cell counting device. A hemacytometer is used in conjunction with a stain, such as trypan blue (MT 25-900-CI). Simply remove a small amount of the cell suspension, such as 500ml, and mix with an equal amount of trypanblue. Using a clean hemacytometer, determine the number of cells/ml in the suspension and calculate the volume of suspension required to seed the desired density for each subculture.

8. Dispense these aliquots into clean, sterile, labeled culture vessels. Add the desired amount of culture medium to the vessels and pipette to ensure equal distribution of cells.

9. Return cultures to their appropriate environment. Most mammalian cell lines require a 37°C growth environment including a carbon dioxide level of 5%. The type of culture medium may alter the type of environment required for cell growth.

10. After about 24hours, observe the culture for reattachment and active growth. Note any unusual observations. Change medium as needed and subculture when necessary.

References:
1. Davis, J.M. *Basic Cell Culture.* Oxford University Press, New York, 93-134 (1996).
2. Doyle, A. and J. Bryan Griffiths. *Mammalian Cell Cuture.* John Wiley & Sons, West Sussex, UK, 47-48 (1997).
3. Freshney, R.I. *Animal Cell Culture: A Practical Approach.* Oxford University Press, New York, 62-64 (1992).
4. Freshney, R.I. *Culture of Animal Cells: A Manual of Basic Technique, 4th Ed.* Wiley Liss, New York, (2000).

Guide to Subculturing Cell Cultures

Morphology Each cell line or cell type expresses different characteristics in terms of growth and appearance in culture. Many cell lines grow as a single sheet monolayer attached to both themselves, and the culture vessel. Other cell types exist as single cells or clumps of cells suspended in the growth medium. Both adherent and suspension cultures must be maintained regularly to prevent overgrowth and accelerated cell death from exhausted medium and to promote the growth of the next generation of cells.

Cell Dissociation Viable subcultures may be obtained by transferring a particular volume of cells to new culture vessels with fresh medium. These fresh cultures are allowed to grow and divide as normal until such time the culture reaches confluence and the cells are used for experiments or subcultured. To do this correctly, obtain a single-cell suspension first. For adherent cell types, proteolytic enzymes, such as trypsin (MT Catalog Numbers 25-050, 25-052, 25-053, and 25-054), are used to break cell-cell and cell-substrate bonds and create a suspension from which new cultures may be split. For cultures already growing in suspension, this enzyme step is not necessary.

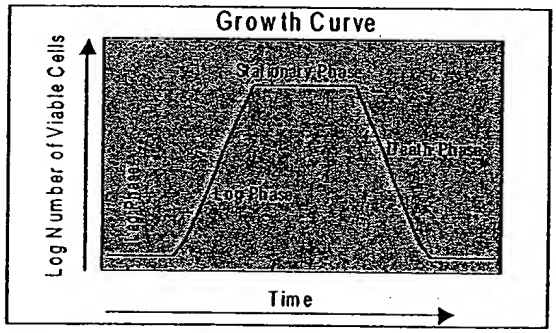
Harvesting describes the detachment of adherent cell lines to prepare a cell suspension for counting. During this step, the intercellular and intracellular (cell-substrate) bonds are broken, allowing the cells to separate into a single cell suspension. Depending on the cell type and the culture environment, this is achieved by using enzymatic or non-enzymatic dissociation solutions, such as Cellstripper®, catalog number 25-056.

Growth Phases Cell growth typically exhibits a consistent pattern comprising three main phases, including an initial lag phase, a period of logarithmic growth, and a final stationary phase. See Figure 1. This growth pattern continues for each subculture despite the cell type. The initial lag phase occurs at the beginning of a subculture as the cells become accustomed to the new environment, during which they do not divide. The length of the log phase is determined by cell conditions prior to subculturing, as well as the seeding density and changes in the growth medium. The log (logarithmic) phase is a period of active proliferation, during which the number of cells increases exponentially. The length of the log phase is determined by many factors, including the seeding density and rate of normal cell growth, as well factors affecting the lag phase. The final stationary phase occurs when the rate of cell proliferation slows down. During this phase, the rate of cell division may be balanced by the rate of cell death, thus showing no change in cell density.

Subculturing is usually performed during the log phase when the cells are at their healthiest and are able to adapt to the new environment most efficiently. This is also the best time for cryopreservation and functionality studies. Check for cultures that appear at least 70% confluent.

Culture Examination Before handling, it is good practice to observe cultures both microscopically and macroscopically. Visual observations of the culture flask and medium may indicate evidence of microbial contamination including pH fluctuations and turbidity, as well as fungal colonies. The monolayer may also be viewed macroscopically to obtain a general idea of confluence. See Figures 2 and 3 below. This is most easily performed by viewing the culture vessel against a light source. Further microscopic observation may substantiate abnormal cell appearance and confirm microbial contamination. Rounding cells may indicate mitosis, especially if the cells are very refractive, or bright. Dead cells do not express this same brightness.

Figure 1. Growth Phases of Cells in Culture



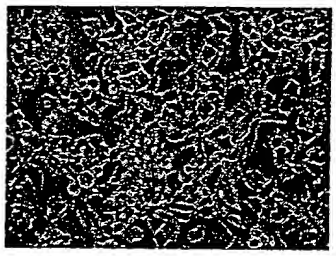


Figure 2. Monolayer
Confluent Monolayer. This culture is ready for splitting.

Figure 3. Monolayer
This monolayer appears less confluent than the monolayer in Figure 1. A cell count would determine if maximum cell density has been reached.

Biochemical Methods in Cell Culture and Virology

ROBERT J. KUCHLER
Rutgers—The State University

Dowden, Hutchinson & Ross, Inc.
STROUDSBURG, PENNSYLVANIA

3

Development of Animal Cell Populations *In Vitro*

The capacity for animal cells to function as independent units was first established when Sanford et al. (1948) isolated a single fibroblast that grew out as a clone. With the development of the plating technique Puck and Marcus (1955) demonstrated that most, if not all, of the cells in a population of an established cell line could develop independently into new clones. Further studies have shown that many cell types removed directly from an animal can also be cloned. From these results, it must be concluded that cells capable of *in vitro* growth function independently, despite the fact that many are interdependent in the intact animal and provide for form and function. The process of animal cell development *in vitro* turns out to be analogous to the growth of microbial populations, and in this chapter we treat it in this manner.

THE GROWTH CYCLE

Populations of animal cells cultured *in vitro* increase in number as the individual cell divides mitotically. Whether they are primary cells, cell strains, or established cell lines, multiplication begins only after a period of adjustment and stops when a number is reached that is saturating for the system. If the logarithm of number of cells present at any time in the culture is plotted as a function of time, a smooth growth curve characteristic for the culture system is obtained (Figure 3-1). The growth curve for mammalian cells can be divided into three phases: the lag phase, the log phase or exponential phase, and the plateau phase (Rinaldini, 1954; Kuchler and Merchant, 1956; Salzman, 1959). The lag phase usually varies from 24 to 48 hours. In this phase, the cells do not divide but are in the process of adapting to the new medium. When the cells begin to divide, the population enters the logarithmic phase of the culture cycle in which the cell number is increasing at a constant rate. During this period, which lasts 2 to 8 days, the population at any time is composed of cells that are at all points in the division cycle. The population doubling time for

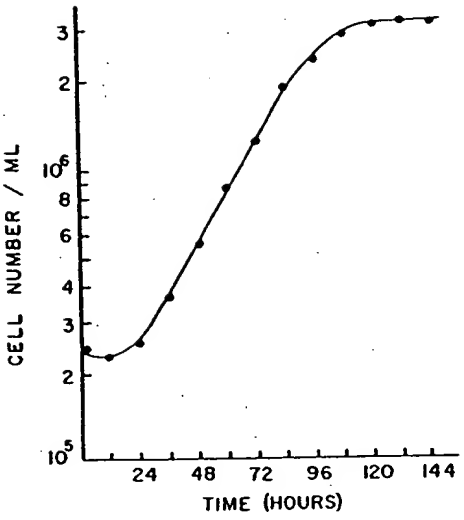


Figure 3-1
Population growth curve for L-M strain mouse fibroblasts in suspension culture.

cultured cells ranges from 12 to 48 hours. Since cultures are usually started with 50,000 to 200,000 cells, four or five population doublings occur during the culture cycle. A population of cells will stop dividing when an essential nutrient is depleted or an inhibitory substance is produced. At this point the culture enters the plateau phase, which is characterized by the fact that a constant cell number prevails over a period of time. The constancy of cell number may result because division ceases in all cells or because some cells degenerate and die while others continue to divide.

Replicate-Culture Technique

Replicate culturing is used to establish a series of identical cultures that can be harvested separately at various times to represent the entire population (Evans et al., 1951; Rinaldini, 1954). In growth studies with cells attached to surfaces, a replicate set of cultures must be used, since a single culture must be harvested for analysis at each time. Cells growing in agitated suspension cultures provide more flexibility, because aliquots can be removed from a single large culture at various times to measure the growth response. The replicate-culture technique is invaluable in quantitative physiological studies where various cultures are subjected to different experimental conditions. Three general procedures for setting up replicate cultures are described.

Manual procedure

Ordinary pipettes can be used to dispense a set of replicate cultures in 1- to 10-ml volumes. The cell pool should be continuously agitated by hand or with a magnetic stirrer.

Automatic procedure

A Cornwall pipetting unit is also useful for distributing small volumes of cell suspension. The intake bulb is immersed in the cell suspension, which is continu-

used as a standard. Ribose between 2 and 20 μg can also be used. Selection of an accurate standard is difficult because the purine-to-pyrimidine ratio of the RNA to be analyzed may not be known.

Protein by the Lowry reaction

The quantitative determination of protein is made by reacting the Folin-Ciocalteau reagent with the alkali-solubilized precipitate after the extraction of DNA and RNA. The blue color that develops is a result of (1) biuret reaction of proteins with copper ion in alkali, and (2) reduction of phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein. Since a cell contains between 300 and 600 pg of protein, the reaction can be carried out using 0.5 to 1.0×10^5 cells.

Reagents

Reagent A: dissolve 0.5 g of $CuSO_4 \cdot 5H_2O$ and 1 g of sodium or potassium tartrate in 100.0 ml of water.

Reagent B: carbonate-copper solution. Mix 50.0 ml of 2 percent Na_2CO_3 with 1.0 ml of reagent A. Discard after 1 day.

Reagent C: alkaline copper solution. Mix 50.0 ml of 2 percent Na_2CO_3 in 0.1 *N* NaOH with 1.0 ml of reagent A. Discard after 1 day.

Reagent D: diluted Folin reagent. Dilute the Folin–Ciochalteau reagent to make it 1 *N* in acid.

Reaction. Add up to 0.2 ml of sample containing 5 to 100 μg of protein and 1.0 ml of reagent B to a test tube. Mix well and allow to stand for 10 minutes or longer at room temperature. Add 0.1 ml of reagent D rapidly with immediate mixing. After 30 minutes or longer, the color is read in a spectrophotometer. For 5 to 25 μg of protein per ml, it is desirable to make the readings at or near the absorption maximum at 750 nm. For more concentrated solutions the readings may be made at 500 nm. Reagent C is used instead of reagent B when proteins are encountered that are difficult to solubilize.

Standard. Bovine serum albumin ranging from 5 to 50 μg is used as the standard protein.

SYNCHRONOUS GROWTH

The sequential order in which biosynthetic events occur during the cell-division cycle can be ideally studied by following the activities in a single cell by cytochemical, autoradiographic, and spectrophotometric methods. Since the same cell cannot be used to follow the events throughout, populations are synchronized, so that representative samples can be removed from the culture at various points in the division cycle. During the past 20 years, some very successful methods have been developed for synchronizing populations of mammalian cells in culture, which, in turn, have led investigators to attempt to map the order of various events occurring during the cell-division cycle. One of the first studies showed that DNA is synthesized in mid-interphase, and is separated by two gaps occurring before and after mitosis (Firket and Verly, 1958). Howard and Pelc (1953) had previously recognized four stage in their studies with bean roots, and named them mitosis (M), first

gap (G1), DNA synthesis (S), and second gap (G2). In mammalian cells, the S stage most often occupies about 7 hours of the division cycle, regardless of the generation time; the M stage occupies 3 to 4 percent of the division cycle. It appears that mammalian cells have different generation times because they vary with respect to the time spent in the G1 stage (Sisken and Kinosita, 1961).

Two general procedures are employed to obtain synchronously growing populations of mammalian cells *in vitro*: (1) a small fraction of the cells in a population can be selectively isolated at a certain point in the division cycle, or the undesired cells can be preferentially destroyed; (2) all the cells, or at least a large fraction, can be blocked at a specific point in the division cycle by using an inhibitory compound, or by withholding an essential nutrient. Procedures representing both approaches are outlined.

Selective Isolation of Synchronously Growing Cells

Collection of loosely attached mitotic cells

Terasima and Tolmach (1963) introduced a simple procedure for the selective isolation of dividing cells; they exploited the observation that cells growing attached to a surface round up during the mitotic period and can be dislodged by using a gentle shearing force. The detached cells are pelleted and resuspended in a complete medium in which they grow in synchrony for one division cycle. A limitation of this method is that only about 4 percent of the cells are in the mitotic stage when the population is growing at an exponential rate, and only about one fourth of these can be obtained. The following procedure can be used to collect dividing cells.

1. Between 1 and 2 X 10^6 cells in 10.0 ml of a complete medium are cultured in a 100-mm plastic petri dish at 37°C in an atmosphere of 5 percent CO_2 in air.
2. The medium is discarded after 18 hours and replaced with fresh prewarmed medium of the same composition. This step is carried out to remove any dead cells that have become detached from the surface.
3. After 6 hours, the medium is again removed and discarded, and 5.0 ml of fresh prewarmed medium is forcibly ejected from a 10.0-ml pipette to wash off the loosely attached dividing cells. The force necessary to release the cells must be determined empirically.
4. The suspension of detached cells is pelleted and resuspended in a fresh or a conditioned medium. Since only small numbers of cells are obtained, the suspension is usually replicated into Leighton tubes containing cover slips.

Separation of uniformly sized cells by gravity

Synchronous cells have been separated by centrifuging exponentially growing populations in linear 2 to 10 percent (w/v) sucrose gradients made up in a complete growth medium (Sinclair and Bishop, 1965). Shall and McClelland (1971) found that cultured animal cells would also stratify according to size in a complete medium under the natural force of gravity. In the latter procedure, the cells selected for their size uniformity had a doubling time of 22 hours, whereas these

Guide to Subculturing Cell Cultures

Morphology Each cell line or cell type expresses different characteristics in terms of growth and appearance in culture. Many cell lines grow as a single sheet monolayer attached to both themselves, and the culture vessel. Other cell types exist as single cells or clumps of cells suspended in the growth medium. Both adherent and suspension cultures must be maintained regularly to prevent overgrowth and accelerated cell death from exhausted medium and to promote the growth of the next generation of cells.

Cell Dissociation Viable subcultures may be obtained by transferring a particular volume of cells to new culture vessels with fresh medium. These fresh cultures are allowed to grow and divide as normal until such time the culture reaches confluence and the cells are used for experiments or subcultured. To do this correctly, obtain a single-cell suspension first. For adherent cell types, proteolytic enzymes, such as trypsin (MT Catalog Numbers 25-050, 25-052, 25-053, and 25-054), are used to break cell-cell and cell-substrate bonds and create a suspension from which new cultures may be split. For cultures already growing in suspension, this enzyme step is not necessary.

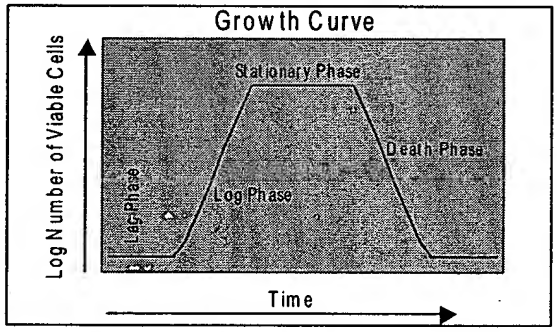
Harvesting describes the detachment of adherent cell lines to prepare a cell suspension for counting. During this step, the intercellular and intracellular (cell-substrate) bonds are broken, allowing the cells to separate into a single cell suspension. Depending on the cell type and the culture environment, this is achieved by using enzymatic or non-enzymatic dissociation solutions, such as Cellstripperä , catalog number 25-056.

Growth Phases Cell growth typically exhibits a consistent pattern comprising three main phases, including an initial lag phase, a period of logarithmic growth, and a final stationary phase. See Figure 1. This growth pattern continues for each subculture despite the cell type. The initial lag phase occurs at the beginning of a subculture as the cells become accustomed to the new environment, during which they do not divide. The length of the log phase is determined by cell conditions prior to subculturing, as well as the seeding density and changes in the growth medium. The log (logarithmic) phase is a period of active proliferation, during which the number of cells increases exponentially. The length of the log phase is determined by many factors, including the seeding density and rate of normal cell growth, as well factors affecting the lag phase. The final stationary phase occurs when the rate of cell proliferation slows down. During this phase, the rate of cell division may be balanced by the rate of cell death, thus showing no change in cell density.

Subculturing is usually performed during the log phase when the cells are at their healthiest and are able to adapt to the new environment most efficiently. This is also the best time for cryopreservation and functionality studies. Check for cultures that appear at least 70% confluent.

Culture Examination Before handling, it is good practice to observe cultures both microscopically and macroscopically. Visual observations of the culture flask and medium may indicate evidence of microbial contamination including pH fluctuations and turbidity, as well as fungal colonies. The monolayer may also be viewed macroscopically to obtain a general idea of confluence. See Figures 2 and 3 below. This is most easily performed by viewing the culture vessel against a light source. Further microscopic observation may substantiate abnormal cell appearance and confirm microbial contamination. Rounding cells may indicate mitosis, especially if the cells are very refractive, or bright. Dead cells do not express this same brightness.

Figure 1. Growth Phases of Cells in Culture



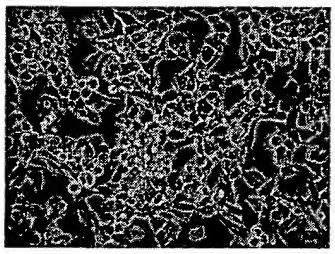


Figure 2. Monolayer
Confluent Monolayer. This culture is ready for splitting.

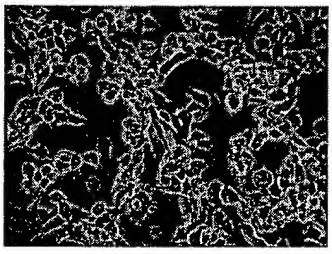


Figure 3. Monolayer
This monolayer appears less confluent than the monolayer in Figure 1. A cell count would determine if maximum cell density has been reached.

Routine Subcultivation & Maintenance

Procedure The following procedure describes the basic principles involved in the routine subcultivation and maintenance of cell cultures. **It is imperative that aseptic technique is maintained throughout this procedure.**

1. Visually examine cultures, as discussed above.

2. Harvest adherent cell lines using a dissociating agent. For suspension lines, this step is not required. Proceed to Step 7.

3. Aspirate and discard the culture medium.

4. Rinse the monolayer with the dissociation solution or a buffered salt solution without calcium and magnesium. This rinsing step removes any residual serum from the monolayer that could inactivate the trypsin. Use about 3ml for 25-cm^2 flasks and 5ml for 75-cm^2 flasks. Be sure to add the solution on the side of the flask opposite the monolayer to prevent cell loss. Swirl the solution gently across the cell sheet. Remove and discard the solution.

5. Repeat the washing step, this time using the dissociation solution. For more fastidious cell lines, the flask can be placed in a 37°C incubator to facilitate the enzyme. The flask may also be observed microscopically to monitor progress and prevent over-exposure of the cells to the enzyme activity. Non-enzymatic solutions, such as Cellstripperä , will require longer incubation times than when using enzymes like trypsin, but are more gentle to the cells.

6. Once the cells are detached, add the desired amount of growth medium to the flask, creating a cell suspension. When pipetting the growth medium into the flask, be sure to "wash" the sides of the flask to ensure all cells become suspended. It is not unusual for a small amount of cells to remain attached to the flask or substrate. However, more vigorous pipetting may be necessary to break up cell clumps or to aid in the removal of attached cells.

7. Using the cell suspension, determine the appropriate inoculum for subculturing the particular cell line. This seeding density may be determined by performing growth rate studies, or by counting. When simply passing a culture, when tracking exact cell densities in not necessary, a split according to a suspension ratio is commonly used. For example, a 1:2 ratio indicates that the cell suspension may be split in half between two culture flasks of equal surface area. Counting, however, requires a hemacytometer or other cell counting device. A hemacytometer is used in conjunction with a stain, such as trypan blue (MT 25-900-CI). Simply remove a small amount of the cell suspension, such as 500ml, and mix with an equal amount of trypanblue. Using a clean hemacytometer, determine the number of cells/ml in the suspension and calculate the volume of suspension required to seed the desired density for each subculture.

8. Dispense these aliquots into clean, sterile, labeled culture vessels. Add the desired amount of culture medium to the vessels and pipette to ensure equal distribution of cells.

9. Return cultures to their appropriate environment. Most mammalian cell lines require a 37°C growth environment including a carbon dioxide level of 5%. The type of culture medium may alter the type of environment required for cell growth.

10. After about 24hours, observe the culture for reattachment and active growth. Note any unusual observations. Change medium as needed and subculture when necessary.

References:
1. Davis, J.M. *Basic Cell Culture.* Oxford University Press, New York, 93-134 (1996).
2. Doyle, A. and J. Bryan Griffiths. *Mammalian Cell Cuture.* John Wiley & Sons, West Sussex, UK, 47-48 (1997).
3. Freshney, R.I. *Animal Cell Culture: A Practical Approach.* Oxford University Press, New York, 62-64 (1992).
4. Freshney, R.I. *Culture of Animal Cells: A Manual of Basic Technique, 4th Ed.* Wiley Liss, New York, (2000).

Biochemical Methods in Cell Culture and Virology

ROBERT J. KUCHLER
Rutgers—The State University

Dowden, Hutchinson & Ross, Inc.
STROUDSBURG, PENNSYLVANIA

Library of Congress Catalog Card Number: 76-48662
ISBN: 0-87933-233-6

79 78 77 1 2 3 4 5

Manufactured in the United States of America.

LIBRARY OF CONGRESS CATALOGING IN PUBLICATION DATA

Kuchler, Robert Joseph, 1928-
Biochemical methods in cell culture and virology
Includes index.
1. Cell culture. 2. Virology—Cultures and culture media. 3. Virology—Technique. I. Title. [DNLM: 1. Tissue culture—Laboratory manuals. 2. Virology—Laboratory manuals. QS525 K95b]
QH585.K83 574.8'7'028 76-48662
ISBN 0-87933-233-6

Exclusive distributor: **Halsted Press,**
A Division of John Wiley & Sons, Inc.
ISBN: 0-470-99043-0

3

Development of Animal Cell Populations *In Vitro*

The capacity for animal cells to function as independent units was first established when Sanford et al. (1948) isolated a single fibroblast that grew out as a clone. With the development of the plating technique Puck and Marcus (1955) demonstrated that most, if not all, of the cells in a population of an established cell line could develop independently into new clones. Further studies have shown that many cell types removed directly from an animal can also be cloned. From these results, it must be concluded that cells capable of *in vitro* growth function independently, despite the fact that many are interdependent in the intact animal and provide for form and function. The process of animal cell development *in vitro* turns out to be analogous to the growth of microbial populations, and in this chapter we treat it in this manner.

THE GROWTH CYCLE

Populations of animal cells cultured *in vitro* increase in number as the individual cell divides mitotically. Whether they are primary cells, cell strains, or established cell lines, multiplication begins only after a period of adjustment and stops when a number is reached that is saturating for the system. If the logarithm of number of cells present at any time in the culture is plotted as a function of time, a smooth growth curve characteristic for the culture system is obtained (Figure 3-1). The growth curve for mammalian cells can be divided into three phases: the lag phase, the log phase or exponential phase, and the plateau phase (Rinaldini, 1954; Kuchler and Merchant, 1956; Salzman, 1959). The lag phase usually varies from 24 to 48 hours. In this phase, the cells do not divide but are in the process of adapting to the new medium. When the cells begin to divide, the population enters the logarithmic phase of the culture cycle in which the cell number is increasing at a constant rate. During this period, which lasts 2 to 8 days, the population at any time is composed of cells that are at all points in the division cycle. The population doubling time for

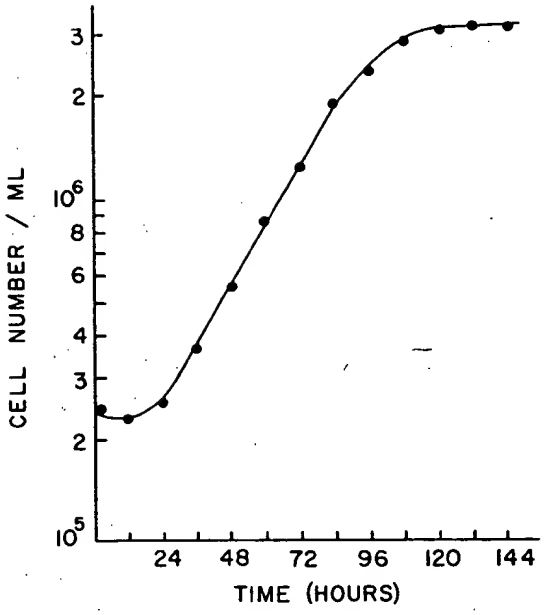


Figure 3-1
Population growth curve for L-M strain mouse fibroblasts in suspension culture.

cultured cells ranges from 12 to 48 hours. Since cultures are usually started with 50,000 to 200,000 cells, four or five population doublings occur during the culture cycle. A population of cells will stop dividing when an essential nutrient is depleted or an inhibitory substance is produced. At this point the culture enters the plateau phase, which is characterized by the fact that a constant cell number prevails over a period of time. The constancy of cell number may result because division ceases in all cells or because some cells degenerate and die while others continue to divide.

Replicate-Culture Technique

Replicate culturing is used to establish a series of identical cultures that can be harvested separately at various times to represent the entire population (Evans et al., 1951; Rinaldini, 1954). In growth studies with cells attached to surfaces, a replicate set of cultures must be used, since a single culture must be harvested for analysis at each time. Cells growing in agitated suspension cultures provide more flexibility, because aliquots can be removed from a single large culture at various times to measure the growth response. The replicate-culture technique is invaluable in quantitative physiological studies where various cultures are subjected to different experimental conditions. Three general procedures for setting up replicate cultures are described.

Manual procedure

Ordinary pipettes can be used to dispense a set of replicate cultures in 1- to 10-ml volumes. The cell pool should be continuously agitated by hand or with a magnetic stirrer.

Automatic procedure

A Cornwall pipetting unit is also useful for distributing small volumes of cell suspension. The intake bulb is immersed in the cell suspension, which is continu-

used as a standard. Ribose between 2 and 20 μg can also be used. Selection of an accurate standard is difficult because the purine-to-pyrimidine ratio of the RNA to be analyzed may not be known.

Protein by the Lowry reaction

The quantitative determination of protein is made by reacting the Folin-Ciocalteau reagent with the alkali-solubilized precipitate after the extraction of DNA and RNA. The blue color that develops is a result of (1) biuret reaction of proteins with copper ion in alkali, and (2) reduction of phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein. Since a cell contains between 300 and 600 pg of protein, the reaction can be carried out using 0.5 to 1.0 X 10^5 cells.

Reagents

Reagent A: dissolve 0.5 g of $CuSO_4 \cdot 5H_2O$ and 1 g of sodium or potassium tartrate in 100.0 ml of water.

Reagent B: carbonate-copper solution. Mix 50.0 ml of 2 percent Na_2CO_3 with 1.0 ml of reagent A. Discard after 1 day.

Reagent C: alkaline copper solution. Mix 50.0 ml of 2 percent Na_2CO_3 in 0.1 *N* NaOH with 1.0 ml of reagent A. Discard after 1 day.

Reagent D: diluted Folin reagent. Dilute the Folin–Ciochalteau reagent to make it 1 *N* in acid.

Reaction. Add up to 0.2 ml of sample containing 5 to 100 μg of protein and 1.0 ml of reagent B to a test tube. Mix well and allow to stand for 10 minutes or longer at room temperature. Add 0.1 ml of reagent D rapidly with immediate mixing. After 30 minutes or longer, the color is read in a spectrophotometer. For 5 to 25 μg of protein per ml, it is desirable to make the readings at or near the absorption maximum at 750 nm. For more concentrated solutions the readings may be made at 500 nm. Reagent C is used instead of reagent B when proteins are encountered that are difficult to solubilize.

Standard. Bovine serum albumin ranging from 5 to 50 μg is used as the standard protein.

SYNCHRONOUS GROWTH

The sequential order in which biosynthetic events occur during the cell-division cycle can be ideally studied by following the activities in a single cell by cytochemical, autoradiographic, and spectrophotometric methods. Since the same cell cannot be used to follow the events throughout, populations are synchronized, so that representative samples can be removed from the culture at various points in the division cycle. During the past 20 years, some very successful methods have been developed for synchronizing populations of mammalian cells in culture, which, in turn, have led investigators to attempt to map the order of various events occurring during the cell-division cycle. One of the first studies showed that DNA is synthesized in mid-interphase, and is separated by two gaps occurring before and after mitosis (Firket and Verly, 1958). Howard and Pelc (1953) had previously recognized four stage in their studies with bean roots, and named them mitosis (M), first

gap (G1), DNA synthesis (S), and second gap (G2). In mammalian cells, the S stage most often occupies about 7 hours of the division cycle, regardless of the generation time; the M stage occupies 3 to 4 percent of the division cycle. It appears that mammalian cells have different generation times because they vary with respect to the time spent in the G1 stage (Sisken and Kinosita, 1961).

Two general procedures are employed to obtain synchronously growing populations of mammalian cells *in vitro*: (1) a small fraction of the cells in a population can be selectively isolated at a certain point in the division cycle, or the undesired cells can be preferentially destroyed; (2) all the cells, or at least a large fraction, can be blocked at a specific point in the division cycle by using an inhibitory compound, or by withholding an essential nutrient. Procedures representing both approaches are outlined.

Selective Isolation of Synchronously Growing Cells

Collection of loosely attached mitotic cells

Terasima and Tolmach (1963) introduced a simple procedure for the selective isolation of dividing cells; they exploited the observation that cells growing attached to a surface round up during the mitotic period and can be dislodged by using a gentle shearing force. The detached cells are pelleted and resuspended in a complete medium in which they grow in synchrony for one division cycle. A limitation of this method is that only about 4 percent of the cells are in the mitotic stage when the population is growing at an exponential rate, and only about one fourth of these can be obtained. The following procedure can be used to collect dividing cells.

1. Between 1 and 2×10^6 cells in 10.0 ml of a complete medium are cultured in a 100-mm plastic petri dish at 37°C in an atmosphere of 5 percent CO_2 in air.
2. The medium is discarded after 18 hours and replaced with fresh prewarmed medium of the same composition. This step is carried out to remove any dead cells that have become detached from the surface.
3. After 6 hours, the medium is again removed and discarded, and 5.0 ml of fresh prewarmed medium is forcibly ejected from a 10.0-ml pipette to wash off the loosely attached dividing cells. The force necessary to release the cells must be determined empirically.
4. The suspension of detached cells is pelleted and resuspended in a fresh or a conditioned medium. Since only small numbers of cells are obtained, the suspension is usually replicated into Leighton tubes containing cover slips.

Separation of uniformly sized cells by gravity

Synchronous cells have been separated by centrifuging exponentially growing populations in linear 2 to 10 percent (w/v) sucrose gradients made up in a complete growth medium (Sinclair and Bishop, 1965). Shall and McClelland (1971) found that cultured animal cells would also stratify according to size in a complete medium under the natural force of gravity. In the latter procedure, the cells selected for their size uniformity had a doubling time of 22 hours, whereas these

CERTIFICATE OF MAILING
37 C.F.R. §1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450, on the date below:

_____ _____
Date

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shuyuan Zhang, Capucine Thwin, Zheng Wu, Toohyon Cho, Shawn Gallagher

Serial No.: 09/203,078

Filed: December 1, 1998

For: METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

Group Art Unit: 1645

Examiner: Shanon A Foley

Atty. Dkt. No.: INRP:081

DECLARATION OF SHUYUAN ZHANG UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

I, Shuyuan Zhang, do declare that:

1. I am a citizen of China residing in the United States at 6015 Briar Hills, Sugarland, Texas, 77479. I am an employee of Introgen Therapeutics, Inc. ("Introgen").

2. I have been employed by Introgen for 6 years and currently hold the position of Associate Director of Production and Process Development at Introgen Therapeutics in Houston,

Texas. I have a Ph.D. in Biochemical Engineering from University of Surrey, England. I have done extensive research and development in the production and purification of retroviral, adenoviral and AAV and non-viral vectors for gene therapy applications. My *curriculum vitae* is attached as Exhibit 1.

3. I am familiar with the level of skill of scientists working in the field of gene therapy as of the October 1992 priority date of the referenced application.

4. I understand that a publication by Leu *et al.*, U.S. Patent 6,194,210, has been cited by the Examiner. I am told that this reference is used by the Examiner to dispute the patentability of claims 1-3, 8-25 and 29 as obvious. For the reasons set forth below, it is my opinion that Leu *et al.* would not by itself or in combination with other references render the present invention obvious. I am told that the Leu *et al.* disclosure is directed exclusively to Hepatitis A virus, a RNA virus and includes no mention whatsoever of adenovirus, a DNA virus that is entirely distinct from the foregoing. Furthermore, I am told that the working examples of Leu *et al.* are directed exclusively to Hepatitis A virus. In light of the facts set forth below, it is evident that one skilled in the art would not look to Leu *et al.* to solve problems relating to propagation of an adenovirus culture in that adenovirus is totally unrelated to Hepatitis A virus and each has numerous distinct properties.

5. Adenoviruses contain double-stranded DNA approximately 36 kb in length. They are covered by a capsid 70-100 nm in diameter. This differs from Hepatitis A viruses in that

Hepatitis A viruses contain positive single-stranded RNA of approximately 7.2–8.4 kb in length. Hepatitis A viruses are comprised of a small, 27-32 nm, protein capsid. The capsid is composed of 60 protein subunits, each consisting of four polypeptides VP1-VP4. The RNA strand is covalently bonded to a noncaspid viral protein (VPg) at its 5' end and to a polyadenylated tail at its 3' end.

6. Mature adenovirus capsid is composed of 252 protein subunits of which 240 are hexons and 12 are pentons. A fiber protein projects from the base of the pentons. The adenovirus core is composed of the linear double stranded DNA and four virus coded core proteins that specifically bind to the DNA. Adenoviruses encode a DNA polymerase but depend on the host cells for many of the other functions involved in synthesis of DNA. DNA replication is complex and distinct from that of other viruses; it involves covalently bound proteins at the ends of the DNA and circularization. Transcription is also complex, involving early and late genes distributed randomly along both strands of the DNA. Replication and assembly occur in the nucleus. After adenoviral replication, the adenovirus is assembled inside the host cell's nucleus resulting in the optimal stability for this virus being at a pH between 7.0-8.5. Hepatitis A viruses on the other hand, are heat- and acid-stable, and relatively detergent resistant. Stability of Hepatitis A viruses is best achieved at a pH of 3.0 or lower. These features of Hepatitis A virus are not shared by adenovirus, and would strongly suggest that purification methods useful for Hepatitis A viruses would not necessarily be relevant to adenovirus.

7. In contrast to adenoviruses, Hepatitis A viruses undergo replication and assembly in the cytoplasm. Their RNA acts as a messenger to synthesize viral macromolecules. Viral RNA replicates in complexes associated with cytoplasmic membranes via two distinct, partially double-stranded RNAs - the "replicative intermediates." One complex uses the sense RNA strand, and the other uses the antisense RNA strand as a template. RNA replication involves the synthesis of a complementary RNA which serves as a template for genome RNA synthesis. Genome RNA also serves as mRNA, being translated into a polyprotein that is cleaved into all the viral proteins including those proteins that serve as enzymes for specific cleavage.

8. Another difference between adenoviruses and Hepatitis A viruses is that the negative DNA strand of the double-stranded DNA of adenoviruses is directly transcribed into viral mRNA, whereas the positive single-stranded RNA of Hepatitis A viruses is copied into negative RNA that is transcribed into viral mRNA.

9. Adenoviruses and Hepatitis A viruses are further distinct in their routes and manner of infection of a cell. Infection of Hepatitis A virus occurs at a specific time in growth from that of adenovirus. Hepatitis A virus infection is nonlytic and usually persists indefinitely. The initial stage of infectivity of Hepatitis A virus occurs during the lag period due to residual inoculum virus which fails to be uncoated. During this stage, viral RNA is released and synthesis of protein and RNA occurs. This is followed by an exponential period at which the concentration of infectivity doubles and leads to a plateau period. Upon infection RNA synthesis decreases in the host cell and subsequently

synthesis of viral RNA in the cytoplasm occurs. Thus, while inhibiting cellular RNA synthesis in the nucleus, viral RNA synthesis in the cytoplasm occurs. This is followed by the inhibition of cellular protein synthesis and rapid synthesis of viral proteins. Following a decrease of viral protein synthesis, leakage of intracellular components occurs leading to cell death.

10. Adenovirus infection is lytic and occurs at high multiplicities. The adenovirus replicative cycle is divided into early and late phases with the late phase beginning at the onset of viral DNA replication. Host cell DNA and protein synthesis are inhibited in cells infected with most adenoviruses as viral DNA synthesis begins. Infection involves the fiber of the virus attaching to a specific receptor on the cell membrane. A decrease in pH alters the surface of the virion resulting in rupture of the endocytic vesicle which releases the virion into the cytoplasm. Adenoviruses, specifically human adenoviruses, remain cell-associated after the production of the new virus is completed. This virus-to-cell association makes it possible for concentration of large quantities of viruses.

11. It is known in the art that both Herpesvirus and Paramyxovirus consist of an envelope with surface projections whereas adenovirus does not have an envelope. Thus, these viruses as compared to adenovirus require an isotonic osmolarity in order to achieve stability and prevent damage to their envelope membrane, whereas adenoviral stability may be achieved at relatively hypertonic osmolarity, as it has no envelope. The replication cycle of Herpesvirus and Paramyxovirus is also distinct from that of adenovirus and involves proteins of the respective envelopes. Following entry into the

host cell these viruses require specific enzymes, thymidine kinase and RNA-dependent RNA polymerase respectively, for transcription. Therefore, the viruses used and mentioned in Leu *et al.* have structural and biological properties distinct from that of adenovirus, and in addition each has a specific time of infectivity from that of adenovirus.

12. The preceding descriptions and comparisons of adenovirus to Hepatitis A viruses, Herpesviruses and Paramyxoviruses provide substantial evidence that, due to the numerous dissimilarities, a teaching relating to one viral type would not necessarily be applicable to the other. Thus, there is no *a priori* expectation that propagation of Hepatitis A viruses or Herpesviruses or Paramyxoviruses would provide appropriate means for adenovirus preparations. In light of these facts, it is my conclusion that the Leu *et al.* patent does not render the above invention obvious.

13. Further, I understand that a publication by Perrin *et al.*, has been cited by the Examiner. I am told that this reference is used by the Examiner to dispute the patentability of claim 4 as obvious. For the reasons set forth below, it is my opinion that Perrin *et al.* would not by itself or in combination with other references render the present invention obvious. I am told that the Perrin *et al.* disclosure is directed exclusively to rabies virus, a RNA virus and includes no mention whatsoever of adenovirus, a DNA virus that is entirely distinct from the foregoing. Furthermore, I am told that the working examples of Perrin *et al.* are directed exclusively to rabies virus. In light of the facts set forth below, it is evident that one skilled in the art would not look to Perrin *et al.* to solve problems

relating to propagation of an adenovirus culture in that adenovirus is totally unrelated to rabies virus and each has numerous distinct properties.

14. Rabies virus and adenovirus are each quite distinct in their structure and biological properties. The rabies virus is an enveloped "budding" RNA-based rhabdovirus whereas adenovirus is a DNA capsid based non-enveloped virus of an entirely different viral family – these viruses infect and grow differently and replicate differently. Thus, there is no *a priori* expectation that propagation of rabies viruses would provide appropriate means for adenovirus preparations. In light of these facts, it is my conclusion that the Perrin *et al.* journal article does not render the above invention obvious.

15. There are a number of scientific publications that support the foregoing conclusion. I would direct the examiner to "Fields Virology" (B. N. Fields, Editor, Vol. 2, 3rd Ed., Lippincott Raven Publishers, 1996; Vol. 1, 4th Ed., Lippincott Williams Wilkins Publishers, 2001), and "The Adenoviruses" (H. S. Ginsberg, Editor, Plenum Press, 1984). I have attached relevant excerpts from these texts as Exhibit 2 to this declaration.

16. I hereby declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the referenced patent application or any patent issued thereon.

02 Mar 04

Date

Shuyuan Zhang, Ph.D